Semiselective Medium for Isolation of *Cryphonectria cubensis*

E. Conradie, W. J. Swart, and M. J. Wingfield

First and second authors, Department of Plant Pathology, and third author, Department of Microbiology and Biochemistry, University of the Orange Free State, Bloemfontein 9300, South Africa.
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ABSTRACT

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Twenty-seven compounds were tested in vitro for their effect on mycelial growth of *Cryphonectria cubensis*, the cause of canker of *Eucalyptus* spp. Eighteen were subsequently tested for their ability to suppress mycelial growth of 15 fungi commonly associated with *C. cubensis* in host tissue. Gallic acid and dicloran, respectively, were the most effective inhibitors of *Trichoderma* sp. and *Mucor* sp. A semiselective medium composed of 20 g of Difco BiTek agar, 10 g of D-glucose, 1 g of L-asparagine, 0.001 g a.i. of chlorothalonil, 0.0055 g a.i. of dicloran, 0.001 g a.i. of vinclozolin, 2 g of gallic acid, 0.1665 g of streptomycin sulphate, and 1 L of distilled water proved very effective for isolating *C. cubensis* from woody tissue and forest soil.

Cryphonectria cubensis (Bruner) C. S. Hodges causes a serious canker of *Eucalyptus* spp. in many tropical areas of the world (2,3,5–8,10,12,16). The recent discovery of *C. cubensis* in South Africa has prompted speculation about possible inoculum sources (16). The fungus may have originated from native Myrtaceae (4), and studies are necessary to determine whether these plants could be a source of inoculum. Similarly, the possible presence of *C. cubensis* inoculum in old tree stumps and in soils where *Eucalyptus* spp. are planted needs to be investigated.

The isolation of *C. cubensis* from soil and diseased wood is severely hampered by the presence of saprophytic fungi and bacteria on these substrates. To date, no selective medium for *C. cubensis* exists that may be used for such a study. This paper describes a semiselective medium for the isolation of *C. cubensis* from both soil and host tissue. Its efficacy is compared with that of a nonselective basal medium.

MATERIALS AND METHODS

Screening of fungicidal activity. Twenty-seven chemical compounds (Table 1) were assayed for their effect on radial growth of a *C. cubensis* isolate obtained from diseased *Eucalyptus* tissue. Each compound was tested at a concentration of 0.1, 0.5, 1, 5, 10, 50, 100, or 500 μg a.i/ml in a basal medium consisting of 20 g of Difco BiTek agar, 10 g of D-glucose, and 1 g of L-asparagine per liter of distilled water. The basal medium was cooled to 50 C before being amended with appropriate volumes of a stock solution of each compound. Media were agitated for 2 min before approximately 20 ml of each concentration was poured into each of five 90-mm petri dishes and allowed to cool. Each plate was seeded with a 5-mm plug taken from the periphery of a 5-day-old potato-dextrose agar (PDA) culture of *C. cubensis*. The plug was placed in the center of the plate, and plates were incubated at 30 C. Fungal growth on the unamended basal medium served as the control. The colony diameter on each plate was determined 5 days later as the mean of two measurements taken perpendicularly to each other. Each test was conducted twice and an analysis of variance (ANOVA) was conducted with the pooled data to compare the effect of different compounds on the growth of *C. cubensis*.

Suppression of test fungi. During screening tests, 18 compounds had an inhibitory effect on *C. cubensis* at concentrations greater than 0.5 μg a.i./ml (Table 1). These compounds were further screened at specific concentrations to determine their effect on mycelial growth of 15 fungi associated with *C. cubensis* in host tissue. Test fungi were *Alternaria alternata* (Fr.:Fr.) Keissl., *Aspergillus niger* Tiegh., *Botryosphaeria* sp., *Botrytis cinerea* Pers.:Fr., *Cryphonectria parasitica* (Murrill) Barr, *Cylindrocladium scoparium* Morg., *Cytospora eucalypticola* Van der Westhuizen, *Endothia gyrosa* (Schwein.:Fr.) Fr., *Epicoccum purpurascens* Ehrenb., *Fusarium* sp., *Graphium* sp., *Mucor* sp., *Pestalotia* sp., *Penicillium* sp., and *Trichoderma* sp. Test fungi were grown on PDA for 4 days at 25 C. A 5-mm plug of agar was transferred from the periphery of each fungal colony to each

TABLE 1. Effect of different fungicides and chemicals on inhibition of radial growth of *Cryphonectria cubensis*[a]

Compound	Lowest concentration causing >50% reduction of growth (μg a.i./ml)	Inhibition of *C. cubensis* (%)
Sportak	0.1	92.1
Etaconazole	0.1	90.4
Bitertanol	0.1	87.6
Terbuconazole	0.1	87.0
Propiconazole	0.1	85.4
Carbendazim	0.1	81.1
Cycloheximide	0.1	71.1
Imazalil	0.1	60.3
Benomyl	0.5	61.6
Novobiocin	1	64.5
Trichlorifon	1	59.9
Chlorothalonil	1	58.1
Pentachloronitrobenzene	1	51.7
Tridemorph	1	51.4
Rose Bengal	5	81.2
Vinclozolin	5	78.7
Iprodione	5	78.4
Procymidone	5	67.3
Dodine	10	80.2
Dicloran	10	62.2
Propineb	50	89.4
Benodanil	50	77.1
o-Phenylphenol	50	76.4
Captab	100	82.3
Etridiazole	500	90.2
Streptomycin sulphate	>500	0.0
Gallic acid	>500	0.0

[a]Reduction in mean colony diameter compared with the basal medium (20 g of Difco BiTek agar, 10 g of D-glucose, 1 g of L-asparagine, and 1 L of water) after 120-h incubation at 30 C. Means based on two measurements (diameters) of each colony and two replicate tests.

of five plates containing the specific compound being screened. Colony diameters were recorded as the mean of two perpendicular measurements after 7 days' incubation at 30 C. The experiment was conducted twice, and an ANOVA was conducted on the pooled data for each compound to compare the growth of *C. cubensis* and the 15 test fungi.

Combination of compounds. Various combinations of compounds either very selective toward *C. cubensis* or highly inhibitory to the two fastest-growing fungi, *Mucor* sp. and *Trichoderma* sp., were incorporated in the basal medium and tested for their combined effect on the growth of *C. cubensis* and the 15 test fungi. Excessive inhibition of *C. cubensis*, presumably resulting from synergism between inhibitors, was alleviated by randomly adjusting the concentration of specific compounds. Each specific combination of compounds was tested twice, and an ANOVA was performed on the pooled data.

Mycelial growth of four other isolates of *C. cubensis* obtained from Brazil, Cameroon, Zanzibar, and Hawaii was compared with that of the South African isolate on the basal and *Cryphonectria* mediums. Colony diameters were measured after 4 and 7 days, and a factorial ANOVA was performed on the pooled data of two trials.

Verification of the semiselective medium. To verify the efficacy of the *Cryphonectria* medium, naturally infected *Eucalyptus* tissue was assayed for the presence of *C. cubensis*. Fifty pieces of tissue (~4 mm^2) from 10 diseased trees were cultured without surface sterilization on *Cryphonectria* medium and the unamended basal medium. Percentage of recovery of *C. cubensis* was then determined.

In a second test, 20 young *E. grandis* plants having stems 15 mm in diameter were artificially inoculated with *C. cubensis*. After approximately 2 wk, when a lesion had developed on each stem, 10 stems were buried in unsterilized forest soil for 4 days. Small pieces of tissue (~4 mm^2) were subsequently removed from each stem and cultured on *Cryphonectria* and basal mediums. For comparative purposes, 50 pieces of tissue from 10 inoculated stems that had not been buried also were cultured on the two media. The test was repeated, and a factorial ANOVA was performed on the combined data of the two trials to compare the mean percentage of recovery of *C. cubensis* on the basal and *Cryphonectria* mediums.

A third test was conducted to determine if *C. cubensis* could be isolated from unsterilized forest soil. Inoculum was prepared by culturing the fungus in 250-ml glass flasks on a mixture of

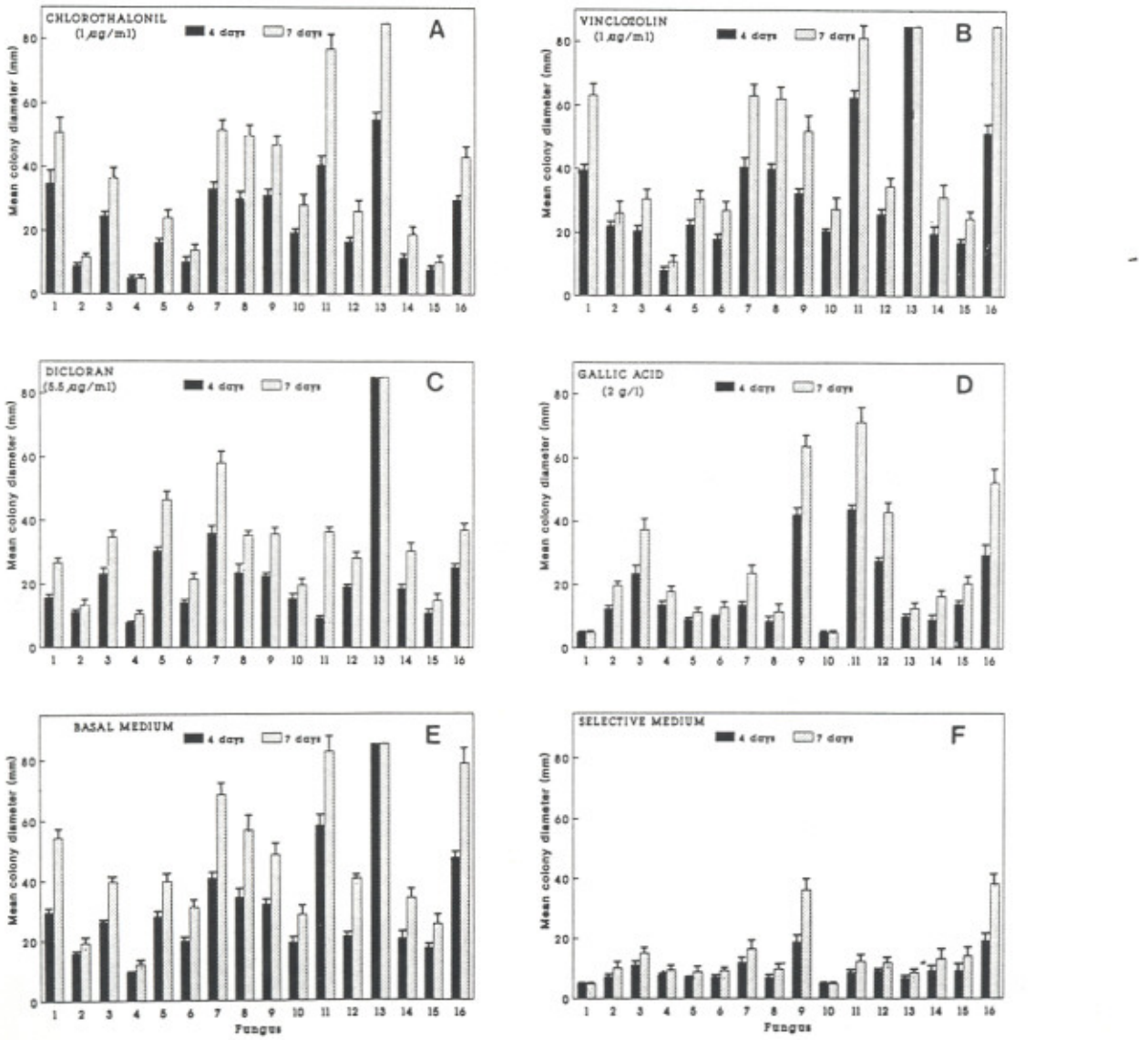

Fig. 1. Mean linear growth of 15 saprophytic fungi and *Cryphonectria cubensis* on basal medium amended with **A,** chlorothalonil, **B,** vinclozolin, **C,** dicloran, **D,** gallic acid, **E,** basal medium, and **F,** *Cryphonectria* selective medium after 4 and 7 days at 30 C. Error bars represent the standard errors of individual means. Fungi are labeled as 1 = *Botryosphaeria* sp., 2 = *Botrytis cinerea*, 3 = *Cylindrocladium scoparium*, 4 = *Cytospora eucalypticola*, 5 = *Fusarium* sp., 6 = *Graphium* sp., 7 = *Pestalotia* sp., 8 = *Alternaria alternata*, 9 = *Aspergillus niger*, 10 = *Epicoccum purpurascens*, 11 = *Mucor* sp., 12 = *Penicillium* sp., 13 = *Trichoderma* sp., 14 = *Endothia gyrosa*, 15 = *Cryphonectria parasitica*, 16 = *C. cubensis*.

sterile sand, maize meal, bran (95:3:2, w/w), and 26 ml of water. Flasks were incubated at 30 C for 10 days. Inoculum was then mixed with moist, freshly collected forest soil at rates of 100, 80, 60, 40, or 20% (w/w). A sample (1 g) of each soil-inoculum mixture was suspended in 15 ml of 2% water agar in a petri plate and allowed to solidify. A 5-mm plug from each mixture was then placed in the center of each of five plates containing *Cryphonectria* or basal medium, and the plates were incubated at 30 C. Fungal colonies that subsequently developed after 7 days were transferred to sterile plates containing PDA, and *C. cubensis* colonies were identified. The experiment was repeated, and a factorial ANOVA was performed on the mean percentage of recovery of *C. cubensis* for the combined trials.

RESULTS

Screening of fungicidal activity. Of the 27 compounds, nine inhibited radial growth of *C. cubensis* by more than 50% at concentrations less than or equal to 0.5 μg a.i./ml (Table 1). These compounds were not used for further screening. The remaining 18 compounds inhibited growth of *C. cubensis* by more than 50% when concentrations greater than 0.5 μg a.i./ml were used.

Suppression of test fungi. Compounds that resulted in *C. cubensis* obtaining a significantly greater colony diameter ($P < 0.05$) than 50% or more of the 15 test fungi were selected for further testing (data not presented). Four compounds, chlorothalonil, dicloran, and, especially, vinclozolin and gallic acid, satisfied this criterion (Fig. 1A–D). The greatest difference in colony diameter of the two fastest-growing fungi, *Trichoderma* sp. and *Mucor* sp., relative to *C. cubensis*, occurred on media containing gallic acid and dicloran, respectively.

Combination of compounds. The combination of chlorothalonil, dicloran, vinclozolin, and gallic acid together with streptomycin sulphate in the basal medium (Fig. 1E) resulted in a semiselective medium, designated *Cryphonectria* medium (Fig. 1F), that allowed *C. cubensis* to attain a greater colony diameter than more than 90% of the 15 test fungi. The concentrations of vinclozolin and dicloran were reduced due to excessive inhibition of *C. cubensis*. The final *Cryphonectria* medium comprised 20 g of Difco BiTek agar, 10 g of D-glucose, 1 g of L-asparagine, 0.1665 g of streptomycin sulphate, 0.001 g a.i. of chlorothalonil, 0.0055 g a.i. of dicloran, 0.001 g a.i. of vinclozolin, and 2 g of gallic acid per liter of distilled water.

Although all five isolates of *C. cubensis* grew significantly slower ($P < 0.01$) on the basal medium than on *Cryphonectria* medium, there was no significant difference in radial growth among the five isolates on the two media.

Verification of the semiselective medium. Isolation from *Eucalyptus* tissue naturally infected with *C. cubensis* yielded 38% *C. cubensis* on *Cryphonectria* medium, compared with 15% on basal. Recovery of *C. cubensis* from buried *Eucalyptus* stems was 30 and 42% on basal and *Cryphonectria* mediums, respectively. Recovery from stems not buried was 95 and 78% on the *Cryphonectria* and basal mediums, respectively.

C. cubensis was not recovered from the inoculum-soil mixture on the basal medium. On *Cryphonectria* medium, however, recovery rates were 80, 50, 30, and 20% from mixtures containing 100, 80, 60, and 40% inoculum, respectively.

DISCUSSION

Numerous media have been developed for the selective isolation of fungi (9,11,13–15). Principles involved in the development of these media include either selective inhibition or enhancement of fungi (14). The semiselective medium developed here is based on both principles. The basal medium contained substances such as glucose and asparagine, which are, respectively, excellent C and N sources for *C. cubensis* (1). *Mucor* sp. and *Trichoderma* sp. grew faster than *C. cubensis* on the basal medium (Fig. 1E), which required their inhibition by means of other compounds.

In developing a selective medium, it is crucial to restrict the growth of rapidly proliferating fungi and fungi that produce masses of easily dispersed spores. In this respect, *Trichoderma* spp., *Mucor* spp., *A. niger*, and *Penicillium* spp. are particularly important, since we frequently isolate these species from eucalypt tissue in association with *C. cubensis*. Chlorothalonil (Fig. 1A) and, especially, gallic acid (Fig. 1D) were effective suppressants of *Trichoderma* sp. and were thus incorporated in the basal medium. Dicloran was most effective inhibitor of *Mucor* sp., and vinclozolin proved to be the best compound for inhibiting the growth of *A. niger* and *Penicillium* sp. relative to that of *C. cubensis*.

Synergism and antagonism among compounds in a selective medium can prevent successful isolation of the target organism (14). For this reason it was necessary to reduce the concentrations at which vinclozolin and dicloran were initially found to inhibit *C. cubensis* (Table 1) so as to prevent undue inhibition of *C. cubensis*. The general growth inhibition of all fungi, including *C. cubensis* on the *Cryphonectria* medium, can probably be attributed to some degree of synergism. Growth of *A. niger*, and to a lesser extent *Penicillium* sp., relative to that of *C. cubensis* and most of the test fungi, was stimulated by gallic acid. This probably accounted for the relatively fast growth of *A. niger* on *Cryphonectria* medium (Fig. 1F). However, this small disadvantage is outweighed by the strong inhibition by gallic acid of the more prevalent and faster-growing *Trichoderma* sp.

The semiselective medium described in this paper provides an improved means of isolating *C. cubensis* in association with microorganisms found in forest soil and woody eucalypt tissue. In addition to its use for routine isolations, it is a potentially valuable tool for ecological studies of *C. cubensis*.

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